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14. ABSTRACT N-acetyltransferase 1 (NAT1) catalyzes N-acetylation of aryl amine carcinogens resulting in their activation or inactivation. NAT1*10, NAT1*11 and NAT1*14, common variant alleles have been epidemiologically associated with increased risk for numerous cancers including breast. NAT1 is also upregulated in breast cancer. We employed a novel approach to study functional differences caused by NAT1*10, NAT1*11, and NAT1*14 polymorphisms by using constructs that mimic complete human mRNAs. Plasmid constructs of NAT1*10 and NAT1*4 contained full length human mRNAs including either the NATa (alternative promoter) or NATb (major promoter) 5'-UTR, the ORF, and 885 base pairs of the 3'UTR region. Following transient or stable transfection into Chinese hamster ovary cells, NAT1-catalyzed N-acetylation of p-aminobenzoic acid was measured by HPLC and NAT1 protein expression was measured by Western blot. mRNA levels were studied using RT-PCR and polyA patterns by RNase Protection. No differences were observed in acetylation activity, protein levels, mRNA, or polyA patterns between NAT1*10, NAT1*11 and NAT1*4, but significant differences were seen between NAT1*14 and NAT1*4. Significant differences were also seen between all constructs containing the NATb 5' UTR compared to those containing the NATa 5' UTR.				
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Introduction

N-acetyltransferase 1 and 2 (NAT) are important phase II metabolic enzymes responsible for the biotransformation of many arylamine compounds including pharmaceuticals and environmental carcinogens. Arylamines can be found as components of cigarette smoke and meat that is cooked at high temperatures. These carcinogens must be bioactivated by metabolic enzymes in the body in order to exert a carcinogenic effect. Arylamines contain exocyclic amines that may be directly *N*-acetylated by NAT. NATs catalyze *N*-acetylation by transferring an acetyl group from acetyl-CoA to the exocyclic nitrogen of an aromatic or heterocyclic amine. These compounds can then be excreted from the body. Heterocyclic amine carcinogens are primarily activated in the body by a two step process. The first step is *N*-hydroxylation carried out by the phase I metabolic enzymes, cytochrome p450s. NAT1 and NAT2 further activate *N*-hydroxy metabolites via *O*-acetylation to form acetoxymetabolites. These acetoxymetabolites quickly form highly electrophilic intermediates that react with DNA to form bulky adducts. If these lesions are not repaired, mutagenesis and carcinogenesis may result. Because polymorphisms in NAT1 are known to alter NAT1 protein level and enzymatic activity, there may be a correlation between NAT1 allele type and cancer risk.

Body

Objective 1: To create pcDNA5/FRT vector constructs that possess the human NAT1 alleles including NATa and NATb 5'-UTR exons, the coding region, and 885 nucleotides of the 3'-UTR (with 6 potential polyadenylation signals). In addition to the reference NAT1*4, alleles possessing individual or combinations of genetic polymorphisms present in NAT1*10, NAT1*11, and NAT1*14 will be constructed.

Polyadenylation Site Removal

The polyadenylation site from the pcDNA5/FRT vector was removed to allow the NAT1 polyadenylation sites to be active and to detect any functional effects of polymorphisms located on NAT1 polyadenylation sites. This was accomplished using restriction endonucleases, Apa I and Sph I (New England Biolabs, Ipswich, MA), followed by ligation with T4 Ligase (Invitrogen).

NATa and NATb NAT1*4 Construction

Two reference NAT1*4 constructs were cloned to mimic the most common transcript originating from each of the two alternative NAT1 promoters, NATa and NATb. mRNAs deriving from the NATb promoter have been detected in nearly all tissues studied, while mRNAs deriving from the NATa promoter are primarily expressed in the liver, kidney, trachea, and lungs (Barker et al, 2006). The NAT1 portion of the construct includes all exons found in the most common NAT1 transcripts originating at the NATa or NATb promoters (Barker et al, 2006). The NATa and NATb constructs have identical coding regions and 3' UTRs. Both constructs include the entire coding region comprised of 873 nucleotides (nts) and 885 nts of the 3' UTR. The only difference in the two constructs is in the 5' region. The NATb construct includes exon 4 and exon 8 comprising 117 nts of the 5' UTR while the NATa construct includes exons 1, 2, 3, and 8 comprising 371 nts of the 5' UTR. Both constructs share a 79 nt exon directly 5' of the coding region termed Exon 8, Exon 9 (the coding region) and the 3' UTR. The 5' segments were amplified from cDNA prepared from NAT1*4/NAT1*4 homozygous RNA isolated from HepG2 cells. The forward primer used to amplify the NATb 5' region was 5'-ATGTGGGAGGATTGCATTCACT-3' while the forward primer used to amplify the NATa 5' region was 5'-AACACATTCTGCTCAAATAAAGCCTA-3'. The reverse primer used to amplify both the NATa and NATb 5' region was

5'-TTCCTCACTCAGAGTCTTGAAGTCTATT-3'. The coding region and 3' UTR were amplified as one piece from *NAT1*4/NAT1*4* homozygous human genomic DNA. The forward primer used to amplify the coding region/3' UTR was 5'-AGACATCTCCATCATCTGGTGTCTTACTAGT-3' and the reverse primer was 5'-ATAGTAGGTACCCTAGCATAAATCACCAATTTCCAAGATAA-3'. The two sections, the 5' UTR and the coding region/3'UTR, were fused together using nested primers. The forward nested primer for NATb 5'-TTTAAAGCTAGCATTCACTAGTTCCTGGTTGCCGGCT-3' and the forward nested primer for NATa was 5'-AACACATTCTGCTCAAATAAAGCCTAGGCCAAACT-3'. The reverse nested primer for both NATa and NATb constructs was 5'-ATAGTAGGTACCTCTGAATTATAGATAAGCAAAGATTCAGATTCT-3'. The pcDNA5/FRT vector and NAT1 allelic segments were incubated with restriction endonucleases Kpn1 and Nhe1. The NAT1 alleles were then ligated into pcDNA5/FRT using T4 ligase. In this report, these two constructs are referred to as NATa 1*4 and NATb 1*4.

NATa and NATb *NAT1*10*, *NAT1*11* and *NAT1*14* Construction

NATa and NATb *NAT1*10* constructs were created using the same NATa and NATb 5'-UTRs amplified from cDNA prepared from *NAT1*4/NAT1*4* homozygous RNA isolated from HepG2 cells, while the coding region and 3' UTR were amplified as one piece from *NAT1*10/NAT1*10* homozygous human genomic DNA. These two sections, the 5' UTR and the coding region and 3' UTR were fused together using nested primers. The same primer sequences were used as described above. Upon sequencing to ensure allelic and junction site integrity, it was discovered that one of the *NAT1*10* clones had 4 additional polymorphisms located in the region 3' to the ORF including 1571T>C, 1642A>C, 1647 Δ CT, and 1716C>T. The presence of these polymorphisms in NAT1 was verified against NCBI databases. This project refers to this allele as *NAT1*10B* and was used to compare *N*-acetylation activity along with *NAT1*10* and *NAT1*4*.

	Nucleotide Position						
Allele	1088	1095	1191	1571	1642	1647	1716
NAT1*4	T	C	G	T	A	CT	C
NAT1*10	A	A	T	T	A	CT	C
NAT1*10B	A	A	T	C	C	Δ CT	T

Table 1: Polymorphisms present in *NAT1*10* and *NAT1*10B*.

NATa and NATb *NAT1*11* constructs were created using the already completed *NAT1*4* constructs. The *NAT1*4* (1,651 nt) polymorphic region was removed from the vector using restriction endonucleases, BstEII and BseRI and the corresponding *NAT1*11* polymorphic region (445G>A, 640T>G, and Δ 9 1065-1090) was amplified from *NAT1*4/NAT1*11* heterozygous human genomic DNA (Coriell) using forward primer 5'-TGTTGAATTCCCTAGAAAAGT-3' and reverse primer 5'-ATAGTAGGTACCCTAGCATAAATCACCAATTTCCAAGATAA-3'. *NAT1*11* DNA was selected using restriction fragment length polymorphism (RFLP) and ligated into the vector using T4 ligase. NATa and NATb *1*14* constructs were created using the previously constructed *NAT1*4* constructs. The region containing the *NAT1*4* polymorphism was removed using restriction endonucleases, AflII and SbfI and the corresponding *NAT1*14* polymorphic region (560G>A) was obtained from a previously constructed *NAT1*14* plasmid using the same

restriction endonucleases and was ligated into the NATa and NATb vectors using T4 ligase. All constructs were completely sequenced to ensure allelic and junction site integrity.

Objective II. Nucleotide excision repair deficient Chinese hamster ovary cells expressing human CYP1A1 will be transfected with pcDNA5/FRT vectors containing human NAT1 constructs. The functional effects of genetic polymorphisms in NAT1*10, NAT1*11 and NAT1*14 will be compared to the reference allele NAT1*4 in transient transfection experiments. Functional assays will include determinations of N- and O-acetylation catalytic activities (HPLC assays), mRNA levels (Taqman assays) and protein (Western blot assays).

Transient Transfections

Transient transfections were performed to determine differences in *N*-acetylation activity between NATa and NATb NAT1 4, NAT1 10, and NAT1 10B. NER-deficient Chinese hamster ovary (UV5/CYP1A1/CHO) cells were transiently transfected with pcDNA5/FRT containing NATa and NATb NAT1*4, NAT1*10, and NAT1*10B allelic constructs using LipofectAMINE plus reagent (Invitrogen) following the manufacturer's recommendations. CHO cells were co-transfected with β -galactosidase on 10 cm plates containing 2.5×10^6 cells. Twenty μ l of lipofectamine plus reagent (Invitrogen), 3.2 μ g NAT1 plasmid DNA, and 0.8 μ g of β -galactosidase transfection control plasmid was diluted in 730 μ l α -minimal essential medium (α MEM) (Lonza, Walkersville, MD). The DNA and lipofectamine mixture was added to the 10 cm plates and allowed to incubate at 37°C for 5 hours then removed and replaced with fresh media. The cells were harvested the next day. Lysate was prepared by centrifuging the cells and resuspending pellet in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 0.1 mM PMSF, and 2 μ g/ml aprotinin). The resuspended cell pellet was subjected to 3 rounds of freezing and thawing and then centrifuged at 15,000xg for 10 minutes. The supernatant was used for enzymatic assays including *N*-acetyltransferase activity and β -galactosidase activity.

To correct for transfection efficiency, β -galactosidase plasmids were cotransfected with pcDNA5/FRT. β -galactosidase activity was measured in reactions containing 30 μ l cell lysate, 70 μ l of 4 mg/ml *ortho*-nitrophenyl- β -D-galactopyranoside (ONPG), and 200 μ l of cleavage buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, and 1 mM MgSO₄, pH 7.0). The reaction was incubated for 30 minutes at 37°C. The reaction was terminated by adding 500 μ l of 1 M sodium carbonate. Absorbance at 420 nm was measured. The β -galactosidase activities were normalized to total lysate protein and the resulting values used to correct for the effect of any differences in transfection efficiency.

Stable Transfections

Stable transfections were carried out in DNA repair deficient (UV5) Chinese Hamster Ovary (CHO) cell lines previously transfected with human CYP1A1 and also containing a single integrated FRT site (UV5/CYP1A1/CHO cell line). The pcDNA5/FRT plasmids containing human NATa or NATb NAT1*4, NAT1*10, or NAT1*10B, were co-transfected with pOG44, a Flp recombinase expression plasmid, into UV5/CYP1A1/CHO cells. pOG44 expresses a temperature-sensitive Flp-recombinase under the control of the human CMV promoter. A synthetic intron is located within the vector to enhance expression of the FLP gene. However, the pOG44 does not remain in the transfected cell line because it contains no antibiotic resistance marker. The Flp recombinase expressed from the pOG44 catalyzes a homologous recombination event between the UV5/FRT cell line and the pcDNA5/FRT expression vector containing the chosen NAT1 DNA. Ten centimeter plates containing 2.5×10^6 CHO cells were co-transfected with plasmid pOG44 and pcDNA5/FRT. Four μ g total of NAT1 and pOG44 plasmid DNA were used in combination with 30 μ l lipofectamine transfection reagent

(Invitrogen) diluted in 730 µl αMEM media. The DNA and lipofectamine mixture was applied to the CHO cells contained in 10 cm plates and allowed to incubate at 37° C for 5 hours then removed and replaced with fresh media. Since the pcDNA5/FRT vector contains a hygromycin resistance cassette, cells were split and passaged through culture media containing 600 µg/ml hygromycin (Invitrogen) to select for cells containing the pcDNA5/FRT with the NAT1 insert. Hygromycin resistant colonies were selected approximately 10 days after transfection and isolated with cloning cylinders.

N-acetyltransferase Enzymatic Activity

Reactions containing 50 µl cell lysate, 40 µl of 300 µM PABA, and 10 µl of 1 mM acetyl coenzyme A were incubated at 37°C for 10 minutes. Reactions were terminated by the addition of 10 µl of 1 M acetic acid. Supernatant was injected into a (125 mm X 4 mm; 5 µm pore size) reverse phase column. Reactants and products were eluted by a Beckman System Gold high performance liquid chromatograph (HPLC) system. *N*-acetyl-PABA was quantitated by absorbance at 280 nm. Measurements were adjusted according to baseline measurements using lysates of the UV5/*CYP1A1*/CHO cell line. Activities were normalized for protein concentration. β-galactosidase activity was used to control for transfection efficiency in transiently transfected cells.

Western Blot

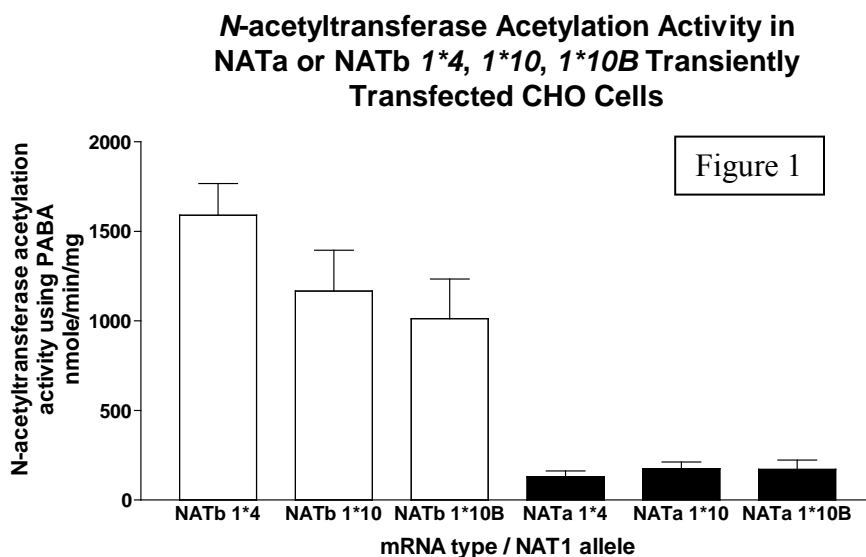
To determine differences in amount of NAT1 protein produced in the transiently or stably transfected CHO cells, western blots were performed. Varying amounts of total protein were used from cell lysates which were collected as described for *N*-acetylation assays and mixed with 2x loading buffer (0.5 M Tris HCl pH 6.8, 10% SDS stock, 2.0 ml/10ml glycerol, 1mg/10ml bromophenol blue), boiled for 5 min, and resolved by 12% SDS-PAGE. The samples were then transferred by semi-dry electroblotting to polyvinylidene fluoride (PVDF) membrane. The membrane was probed with primary polyclonal rabbit anti-hNAT1 antibody ES195 (1:1000, provided by Edith Sim) specific for NAT1 and with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG antibody (1:10,000) (Pierce, Rockford, IL). ECL plus chemiluminescence was used to detect the signal (Amersham Biosciences, Piscataway, NJ).

Relative NAT1 mRNA Detection

TaqMan assays were used to assess the amount of NAT1 mRNA. TaqMan Universal Master Mix (Applied Biosystems, Foster City, California, USA), primers and probes, 96 well optical plates and caps were used. Primers and probes were designed using Primer Express 1.5 software (Applied Biosystems). Synthesis of first strand cDNA was carried out using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's protocol using 1 µg of DNase-treated total RNA. PCR with 1x final concentration of TaqMan Universal Master Mix, 300 nM of each primer and 100 nM of probe in 20 µl total volume was performed using the ABI 7700 sequence detection system (Applied Biosystems). For quantitative RT-PCR of NAT1 mRNA, forward primer 5'-GAATTCAAGCCAGGAAGAAGCA-3' and reverse primer 5'-TCCAAGTCCAATTTGTTCTAGACT-3' was used with TaqMan probe 6FAM-5'-CAATCTGTCTTCTGGATTAA-3'MGBNFQ.

NAT1*10 Transient Transfection Results

Figure 1. Each bar represents 3 separate transfections done in triplicate and error bars represent standard error. All cells transfected with NATb constructs resulted in significantly higher (5.5 – 9.5 fold) *N*-acetylation enzymatic activity. Significance was determined using an one-way ANOVA ($p < .0001$). However, there were no significant ($p > 0.05$) differences in enzymatic activity between NAT1*4 and NAT1*10 transfected cells. Due to the differences in enzymatic activity between the NATa and NATb constructs, western blots were performed to show any differences in protein expression.



NATa and NATb NAT1 10 vs NAT1 4
Western Blot

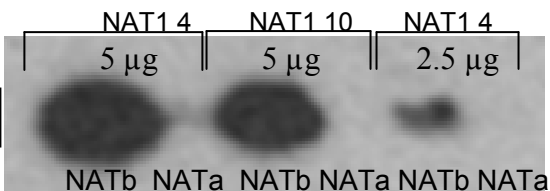


Figure 2. Total protein (5 µg or 2.5 µg) was loaded for western blot analysis. When equal amounts of total protein were loaded, no bands were visible for lysates isolated from CHO cells transiently transfected with NATa constructs, but there were large bands for cells transfected with NATb constructs.

NATa and NATb NAT1 10 vs NAT1 4
Western Blot

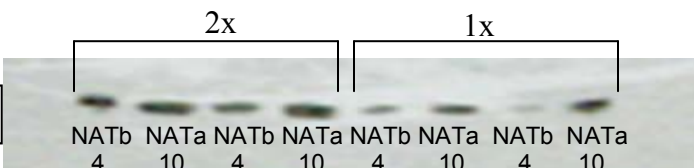


Figure 3. Total protein load amounts were adjusted based on *N*-acetyltransferase enzymatic activity. Similar band intensities were observed when equivalent amounts of enzymatic activity were loaded.

Due to the differences in *N*-acetyltransferase enzymatic activity and protein expression, mRNA was also collected to determine if any differences were present. Total RNA was isolated from collected cells, reverse transcription was performed to create cDNA, and real-time PCR was done to determine relative amounts of mRNA in cells transfected with NATa and NATb *NAT1*4*, *NAT1*10*, and *NAT1*10B*.

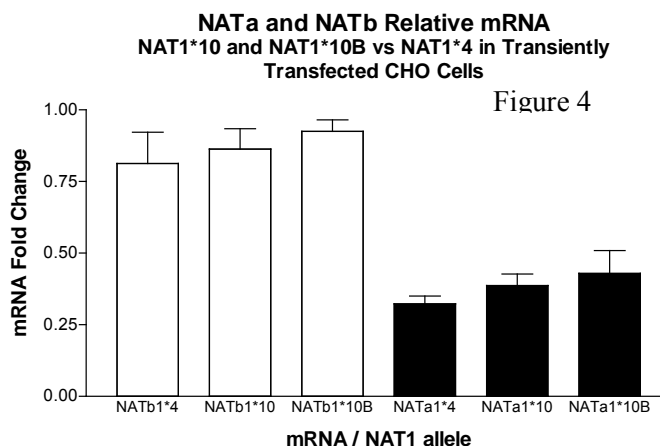


Figure 4

Figure 4. Each bar represents one transfection performed in triplicate and error bars represent standard error. As with enzymatic activity and protein expression determined by western blots, there was significantly more NAT1 mRNA in cells transfected with any NATb construct over cells transfected with any NATa construct. Significance was determined using an one-way ANOVA ($p < .0001$). There were no significant ($p > 0.05$) differences observed between *NAT1*4* and *NAT1*10* transfected cells.

Because of the significant differences between NATa and NATb in transiently transfected cells, stable transfectants were made to be able to study mutagenesis and DNA adduct formation following exposure to environmental carcinogens. Enzymatic activity, protein expression, and mRNA levels were also studied in these stable transfectants.

NAT1*10 Stable Transfection Results

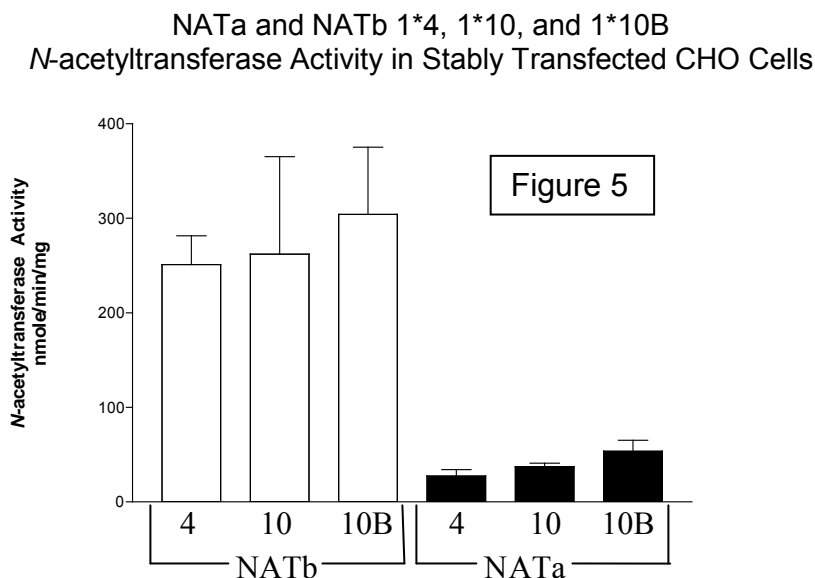


Figure 5

Figure 5. P-aminobenzoic (PABA) assay using HPLC was used to examine NAT1 enzymatic activity performed on stably transfected Chinese hamster ovary cells. Activity values were normalized by total protein concentration. Each bar represents mean ($n=4$) \pm SEM. A range of 5.7 – 9.3 fold more NATb enzymatic activity was detected than NATa. Significance was determined using an one-way ANOVA ($p = .002$). No significant ($p > 0.05$) differences were observed between *NAT1*4*, *NAT1*10*, or *NAT1*10B* allelic constructs.

NATa and NATb NAT1 4, NAT1 10, and NAT1 10B
Western Blot
Figure 6

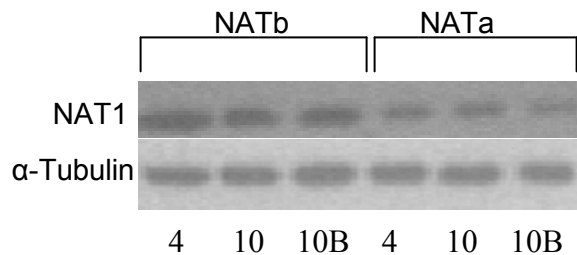


Figure 6. Western blots were performed with cells collected from stably transfected CHO cells to examine protein expression. Twelve μ g of total protein was loaded and densitometric analysis was performed. A range of 3.4 – 4.2 fold greater NATb protein was observed than NATa protein. Significance was determined using an unpaired t-test ($p < .05$). No significant ($p > 0.05$) difference between NAT1 4 versus NAT1 10 protein amount was observed.

Figure 7. Total RNA was collected and a reverse transcription reaction was performed followed by real-time PCR. Each bar represents 3 separate collections of cells tested in triplicate and the error bars represent standard error. Significantly higher (4.2 – 9.0 fold) RNA was detected in cells transfected with NATb constructs over cells transfected with NATa constructs. Significance was determined using a one-way ANOVA ($p = 0.0022$).

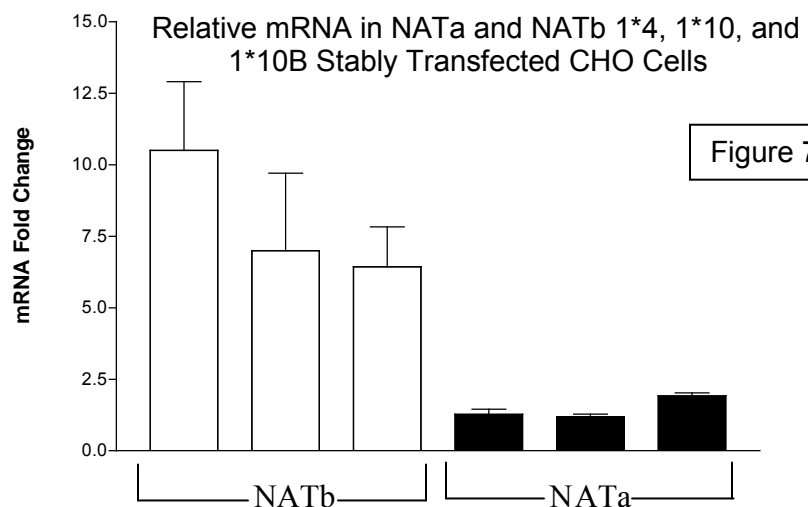


Figure 7

NAT1*11 Transient Transfection Results

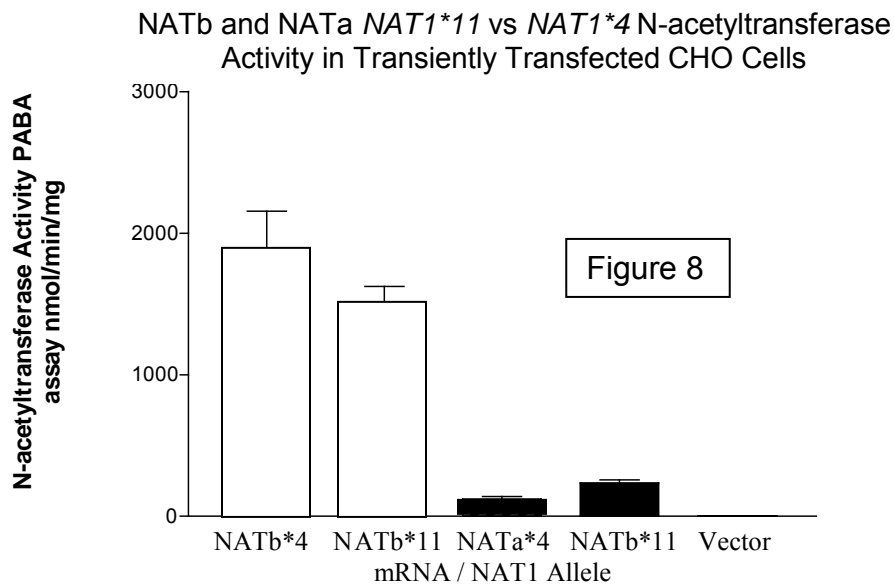


Figure 8. P-aminobenzoic (PABA) assay using HPLC was used to examine NAT1 enzymatic activity performed on transiently transfected Chinese hamster ovary cells. Activity values were normalized by β -galactosidase expression and total protein concentration. Each bar represents mean \pm SEM for three transfections performed in triplicate. A range of 6.5 – 9.2 greater activity was observed for cells transfected with NATb*4 or *11 constructs than NATa*4 or *11. Significance was determined using a one-way ANOVA ($p=.006$).

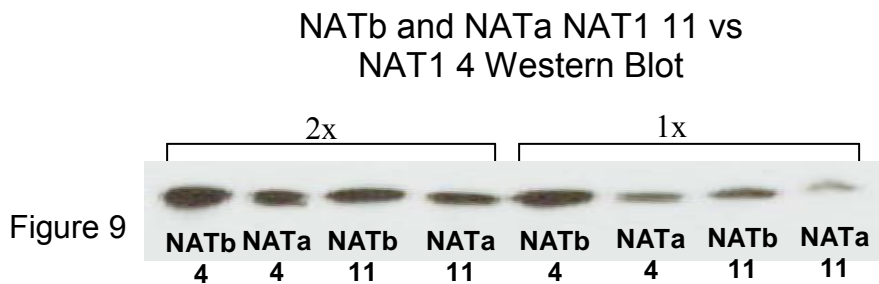


Figure 9. Western blots were performed to examine NAT1 protein expression. Equal amounts of enzymatic activity were loaded. Because different amounts of total protein were loaded, a house-keeping protein could not be probed for so 2x amounts were also loaded to serve as the control. Similar band densities were observed for all allelic variants.

NAT1*14 Stably Transfected Results

Figure 10. P-aminobenzoic (PABA) assay using HPLC was used to examine NAT1 enzymatic activity performed on stably transfected Chinese hamster ovary cells. Each bar represents mean \pm SEM for three separate collections. Approximately 4.5 – fold greater activity was observed for cells transfected with *NATb* 1*14 than *NATa* 1*14. Significance was determined with an unpaired t-test ($p=0.015$).

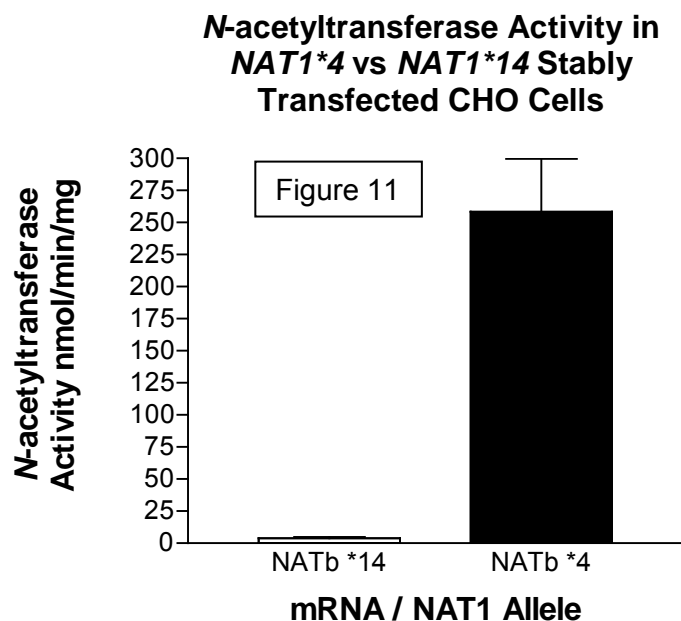
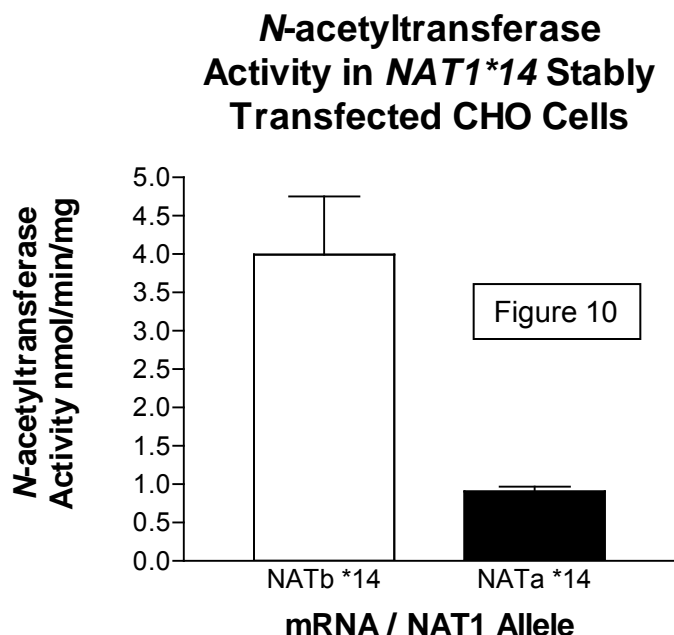


Figure 11. Cells transfected with *NAT1**14 resulted in significantly lower *N*-acetyltransferase activity than cells transfected with the referent allele, *NAT1**4. Significance was determined using an unpaired t-test ($p<.0001$).

Key Research Accomplishments

- Cells transiently and stably transfected with NATb constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*, *1*14*) resulted in significantly higher levels of *N*-acetyltransferase activity than cells transfected with NATa constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*, *1*14*) ($p < .002$).
- Cells transiently and stably transfected with NATb constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*) resulted in significantly higher NAT1 protein levels than cells transfected with NATa constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*) ($p < .05$).
- Cells transiently and stably transfected with NATb constructs (*NAT1*4*, *1*10*, *1*10B*) resulted in significantly higher mRNA levels over cells transfected with NATa constructs (*NAT1*4*, *1*10*, *1*10B*) ($p < .0001$).
- Cells transfected with the variant, *NAT1*14* allele, resulted in significantly less *N*-acetyltransferase activity than cells transfected with the referent allele, *NAT1*4* ($p < .0001$).

Reportable Outcomes

1. Lori M. Millner, David F. Barker, Ashley L. Howarth, Mark A. Doll, J. Christopher States, David W. Hein. Translational Effects of Alternative NAT1 mRNA Isoforms. AACR Frontiers in Cancer Prevention Research International Conference, Washington, DC. November 16-19, 2008 (Abstract).
2. Lori Michele Millner, David F. Barker, Mark A. Doll, J. Christopher States, and David W. Hein. Functional effects of *N*-acetyltransferase 1 (*NAT1*10*) polymorphisms. *FASEB J.* 2009 23:LB394 (Abstract).
3. Lori M. Millner, David F. Barker, Mark A. Doll, J. Christopher States and David W. Hein. Functional Effects of Alternative *N*-acetyltransferase (*NAT1*10*) mRNA Isoforms. Environmental Health Sciences Showcase, NIEHS, University of Cincinnati, Cincinnati, Ohio. September 25, 2009 (Abstract).

Conclusions

Cells transfected with NATb constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*, *1*14*) resulted in significantly higher levels of *N*-acetyltransferase 1 activity ($p < 0.002$), NAT1 protein levels ($p < .05$), and mRNA levels ($p < .0001$) than cells transfected with NATa constructs (*NAT1*4*, *1*10*, *1*10B*, *1*11*, *1*14*). Because the differences in enzymatic activity and protein levels correspond to different amounts of mRNA, there is likely a difference in transcriptional regulation. Since mRNA deriving from the NATa promoter is primarily found in tissues with the highest amount of environmental exposure (kidney, trachea, liver and lungs) (Barker et al., 2006), differential regulation could occur at the transcriptional level to allow for different responses between NATa mRNA and NATb mRNA. More studies need to be conducted to define how transcription is regulated.

Cells transfected with the variant *NAT1*14* resulted in significantly decreased amounts of *N*-acetyltransferase activity ($p<.0001$) than cells transfected with the referent allele, *NAT1*4*. *NAT1*14* is associated with an increased risk of smoking-induced lung cancer. The OR for smoking induced lung cancer was 4.0 (95% confidence interval 0.8-19.6) for homozygous normal (*NAT1*4* or *NAT1*3*) acetylators, whereas the risk was found to be 11.0 (95% confidence interval 1.3-106.5) for heterozygous slow (*NAT1*14* or *NAT1*15*) acetylators (Bouchardy et al., 1998). These studies may increase our understanding of *NAT1*14* and the associated increased cancer risk for smoking induced breast cancer as they have for smoking induced lung cancer.

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